Sulfated fucans and a sulfated galactan from sea urchins as potent inhibitors of selectin-dependent hematogenous metastasis

Teixeira, Felipe C O B ; Kozlowski, Eliene Oliveira ; Borsig, Lubor ; et al

Abstract: Metastasis is responsible for the majority of cancer-associated deaths, though only a very small number of tumor cells are able to efficiently complete all the steps of that process. Tumor cell survival in the bloodstream is one of the limiting aspects of the metastatic cascade. The formation of tumor cell-platelet complexes that promote tumor cell survival is facilitated by the binding of P-selectin on activated platelets to sialyl Lewis-containing oligosaccharides on the surface of tumor cells. Inhibition of this interaction has been shown to attenuate metastasis. Heparin is a potent selectin inhibitor and is capable to block platelet-tumor cell complex formation, thereby attenuating metastasis. Similarly, other sulfated polysaccharides isolated from marine invertebrates attenuate metastasis by a P-selectin-mediated mechanism. In this work, we investigated the selectin-dependent antimetastatic activity of sea urchin sulfated polysaccharides with slight structural differences: a sulfated fucan from Strongylocentrotus franciscanus; a sulfated fucan from Strongylocentrotus droebachiensis; and a sulfated galactan from Echinometra lucunter. The results demonstrate that these fucans and the galactan have different antiselectin activities despite being very similar molecules. Therefore, they may be interesting tools for studies on the structure-function relationship or even for future treatments.

DOI: https://doi.org/10.1093/glycob/cwy020

Sulfated fucans and a sulfated galactan from sea urchins as potent inhibitors of selectin-dependent hematogenous metastasis.

Felipe C. O. B. Teixeira[1], Eliene Oliveira Kozlowski[1,†], Ana Cristina E. S. Vilela-Silva[2], Lubor Borsig[3] and Mauro S. G. Pavão[1,4*]

Key words: sulfated fucan / sulfated galactan / antimetastatic activity / p-selectin

[1] Laboratório de Bioquímica e Biologia Celular de Glicoconjugados, Programa de Glicobiologia, Instituto de Bioquímica Médica Leopoldo de Meis and Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, CEP 21941-913, Brasil

[2] Laboratório de Atividade Biológica de Glicoconjugados, Programa de Glicobiologia, Instituto de Ciências Biomédicas and Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Cep 21941913, Brasil

[3] Institute of Physiology, University of Zurich and Zurich Center for Integrative Human Physiology, Zurich 8057, Switzerland

[4] Instituto de Pesquisas Biomédicas, Hospital Naval Marcílio Dias, Rio de Janeiro, Cep 20725-090, Brasil

[†] Deceased

Running title: Sulfated fucans and galactans have anti selectin activity

* Address correspondence to: Mauro S.G. Pavão, Laboratório de Bioquímica e Biologia Celular de Glicoconjugados, Hospital Universitário Clementino Fraga Filho, Rua Rodolpho Paulo Rocco 255, 4º andar, sala 4A-08, Cidade Universitária, Rio de Janeiro, CEP 21941 913 - Rio de Janeiro – Brazil. Phone: +55-21-3938-2093; Email: mpavao@hucff.ufrj.br

Abstract

Metastasis is responsible for the majority of cancer-associated deaths, though only a very small number of tumor cells are able to efficiently complete all the steps of that process. Tumor cell survival in the bloodstream is one of the limiting aspects of the metastatic cascade. The formation of tumor cell-platelet complexes that promote tumor cell survival is facilitated by the binding of P-selectin on activated platelets to sialyl Lewis-containing oligosaccharides on the surface of tumor cells. Inhibition of this interaction has been shown to attenuate metastasis. Heparin is a potent selectin inhibitor and is capable of blocking platelet-tumor cell complex formation, thereby attenuating metastasis. Similarly, other sulfated polysaccharides isolated from marine invertebrates attenuate metastasis by a P-selectin-mediated mechanism. In this work, we investigated the selectin-dependent antimetastatic activity of sea urchin sulfated polysaccharides with slight structural differences: a sulfated fucan from *Strongylocentrotus franciscanus*; a sulfated fucan from *Strongylocentrotus droebachiensis;* and a sulfated galactan from *Echinometra lucunter*. The results demonstrate that these fucans and galactan have different anti-selectin activities despite being very similar molecules and show potential for future treatments and structure-function relationship studies.

Introduction

Sulfated fucans and sulfated galactans are high molecular weight polyanionic molecules. They are mainly isolated from marine invertebrates and algae. The basic composition of these molecules consists of O-sulfated α-L-fucopyranose (Fuc*p*) or α-L-, α-D-, β-D-galactopyranose (Gal*p*), respectively (Pomin 2012). These polysaccharides are composed of repetitive oligosaccharide units with sulfation patterns that are well-defined, especially those isolated from sea urchins (Pomin 2015). This structural feature is rare among other well-described sulfated polysaccharides, which permits diverse structure-function studies. In fact, many applications in medicine and biomedical research have been reported for these compounds, mainly in coagulation and fertilization (Pereira et al. 2002; Vilela-silva et al. 2008). Recently, similar classes of polysaccharides from marine sources have also described effects on hemostasis, immune modulation and tumor biology (Fitton et al. 2015; Mourão 2015; Fernando et al. 2016), which suggests more extensive medical applications for these molecules.

Tumor progression is an integrative process that involves not only cancer cells but also the tumor microenvironment and stromal cells. The capacity of tumor cells to overcome immunological barrier and reach other sites of colonization is essential for metastasis (Zeeshan and Mutahir 2017). Hematogenous metastasis is one of the most important pathways for cancer progression, and its efficiency is associated with more than 90% of cancer-related deaths (Valastyan and Weinberg 2011). However, shear forces and the presence of immune cells in the bloodstream provide an adverse environment that results in attenuated metastasis. Tumor cells can activate platelets and coagulation, leading to the formation of a tumor cell-platelets-fibrin clot that protects the tumor cells from physical stress and immune surveillance (Borsig et al. 2001). These tumor microemboli are dependent on the platelets' P-selectin expression, and the absence of tumor-derived glycans or the inhibition of P-selectin has been shown to attenuate metastasis (Kim et al. 1998; Ludwig et al. 2007).

Selectins are a family of transmembrane glycoproteins that are involved in carbohydrate-mediated cell adhesion and expressed by many cell types. P-selectin is mainly stored within the Weibel-Palade bodies of endothelial cells and in platelet α-granules, thus allowing a rapid presentation upon activation (Kansas 1996). All members of the selectin family recognize the core tetrasaccharide sialyl-Lewis^{x} (sLe^{x}) and its isomer sLe^{a}, whereas the protein and carbohydrate backbone to which this

ligand is conjugated dictates the specific affinity to the selectins (Kansas 1996). Abnormal glycosylation of cancer cells is considered to be one of the most significant changes in the enhancement of the efficiency of tumor progression. The sialylation of membrane-bound mucins, which raises the affinity to P-selectin, is a striking example (Kim and Varki 1997).

Platelets directly interact with tumor cells via P-selectin during hematogenous dissemination, which increases the potential for the tumor cells to reach a distant site (Leblanc and Peyruchaud 2016). Furthermore, since platelets can interact with the endothelium (Ruggeri and Mendolicchio 2007), platelet-tumor cell emboli formation is also important to ensure the effective arrest in capillaries at metastatic sites and to facilitate the extravasation of the tumor cells (Schumacher et al. 2013).

Heparin is a glycosaminoglycan (GAG) that is known to inhibit P-selectin and, consequently, metastasis. However, due to its high anticoagulant activity, it can potentially cause hemorrhage, which greatly limits its use as an antimetastatic drug (Borsig 2010). Therefore, the search for other sulfated polysaccharides with high anti P-selectin activity and an inhibitory effect on metastasis is an interesting area of investigation. Marine invertebrates are a rich source of heparin-like molecules and sulfated polysaccharides that have high therapeutic potential (Pavão 2014). Although several studies have been carried out on the pharmacological activity of heparin analogs from marine invertebrates, less is known about the anti P-selectin activity of sulfated fucans and sulfated galactans from sea urchins.

Most sulfated fucans and sulfated galactans isolated from the egg jelly of sea urchins have large, simple linear structures, but they can vary in the pattern of sulfation and the position of glycosidic linkage. As shown in Figure 1, the very similar sulfated polysaccharides isolated from *Echinometra lucunter*, *Strongylocentrotus droebachiensis* and *Strongylocentrotus franciscanus* are composed of 2-O-sulfated monosaccharide units that vary both in their glycosidic linkages and their constituent monosaccharides. Whereas *E. lucunter* contains 3-linked α-L-Gal*p*, *S. droebachiensis* and *S. franciscanus* contain 4-linked and 3-linked α-L-Fuc*p* molecules, respectively. The average molecular mass of the *S. droebachiensis* fucan is 80 kDa, while the other two are 100 kDa (Pereira et al. 2002). Despite their similarities, these molecules can ensure species-specificity in sea urchin fertilization, and they also have different anticoagulant effects (Pomin and Mourão 2008).

This work aimed to investigate whether specific features of the chemical structure of sulfated fucans and galactans from sea urchins could affect their ability to prevent selectin-mediated metastasis. For this purpose, the very similar sulfated fucans from *S. droebachiensis and S. franciscanus* and the galactan from *E. lucunter* were tested in mouse models of P-selectin-dependent tumor progression and inflammation.

Results

Sulfated polysaccharides inhibit tumor cell binding to P-selectin

Recent reports have shown the ability of sulfated polysaccharides to inhibit P-selectin binding to its ligands (Gomes et al. 2015; Kozlowski et al. 2011). This is crucial for the antimetastatic activity of these compounds, which not only prevents colonization of distant sites but also extends the patient's lifespan in many types of cancer (Borsig et al. 2007). Due to the similar structures of the three sea urchin sulfated polysaccharides, we assessed their potential to inhibit the adhesion of a human colon adenocarcinoma cell line (LS180) to immobilized P-selectin *in vitro*. Figure 2 shows the representative curves of P-selectin inhibition with increasing concentrations of the sea urchin sulfated polysaccharides and unfractioned heparin (UFH), which was used as a positive control. All glycans inhibited the binding of the tumor cells to the immobilized P-selectin in a dose-dependent manner. In particular, the inhibitory activities of the fucan from *S. droebachiensis* and the galactan from *E. lucunter* were more potent (IC_{50}= 11 and 9,6 µg/mL, respectively) than that for UFH (IC_{50}= 24,5 µg/mL). On the other hand, the fucan from *S. franciscanus* only inhibited the binding at significantly higher concentrations (IC_{50}= 170 µg/mL).

Sea urchin sulfated polysaccharides prevent tumor cell association with platelets in vivo

P-selectin-mediated binding of platelets to the surface of the tumor cells is one of the most important steps for successful hematogenous metastasis. Without the tumor cell-platelet complex, the tumor cells become exposed to physical stress and are susceptible to the immune cells in the bloodstream, which results in reduced metastasis. To determine whether sea urchin compounds can prevent the formation of the tumor cell-platelet complex *in vivo*, calcein-labeled mouse lung

adenocarcinoma cells (LLC) were injected into C57BL/6 mice via the tail vein after injection of the polysaccharides. The interaction of the tumor cells with platelets was quantified via anti-CD41 immunofluorescence in lung sections excised 30 minutes after this procedure. As shown in Figure 3, in animals injected with the *E. lucunter* galactan, 24% of the tumor cells in the pulmonary capillaries were associated with platelets, whereas in those injected with the *S. droebachiensis* fucan, the fraction was 27%. On the other hand, in the animals that had been injected with PBS, 69% of the tumor cells were present in the platelet complexes. These numbers are consistent with the results obtained *in vitro*. As expected from the previous result, the *S. franciscanus* fucan did not significantly reduce this association.

Fucan from S. franciscanus does not present antimetastatic activity

Since these polysaccharides demonstrated potential to inhibit P-selectin and platelet-tumor cell aggregation and given the major importance of these two factors in metastasis, we decided to determine whether the sulfated polysaccharides had any effect on tumor cell metastatic potential. To investigate this, C57BL/6 mice were injected with the polysaccharides, followed by injection of LLC cells. After 21 days, the lungs were harvested and the macrometastasis quantified. Figure 4 shows that treatment with the *E. lucunter* galactan or the *S. droebachiensis* fucan completely prevented metastasis in our model. As expected, the *S. franciscanus* fucan had no antimetastatic activity, with an average of 3,5 metastatic foci per mouse versus 5.6 in the control group.

Sea urchin polysaccharides prevented P-selectin dependent inflammatory cell recruitment

Activated endothelial cells express surface P-selectin, which binds to leukocytes and is essential for cellular recruitment to the inflamed sites (Mayadas et al. 1993). Since our compounds demonstrated a P-selectin dependent antimetastatic activity, we decided to evaluate whether they have effects on P-selectin dependent mediated leukocyte recruitment in an inflammatory model, using a thioglycolate induced acute peritonitis model in mice. After 3 hours of inflammatory stimulus, differential counting of leukocytes was performed in the peritoneal wash of mice and the polymorphonuclear leukocytes (PMN) were quantified. Figure 5 shows that, in accordance with the

previous results (Figures 2 to 4), the *S. franciscanus* fucan was not able to prevent this recruitment and therefore has no significant anti P-selectin activity. On the other hand, *E. lucunter*'s galactan and *S.droebachiensis*' fucan both had a powerful effect. Taken together these data strongly suggest that these polysaccharides block selectin interaction and thereby inhibit metastasis and inflammation.

Discussion

Sulfated polysaccharides include a diverse set of naturally occurring molecules that have been implicated in numerous therapeutic effects. However, the interest in sulfated fucans and sulfated galactans and their applications in medicine has increased strongly only in the past few years. The development of drugs based on naturally occurring carbohydrates is the primary goal of these projects, mainly because of their wide-ranging benefits to human health but also because of their relatively simple chemical structure.

In marine invertebrates, these sulfated fucans and galactans are components of the extracellular matrix. In contrast to those extracted from ascidians and sea cucumbers, which are usually components of their tunic and body wall, respectively (Pavão et al. 1989; Mulloy et al. 1994), the sulfated polysaccharides from sea urchins form a complex extracellular matrix in the jelly layer surrounding the egg, in which they interact with many proteins (Vacquier and Moy 1997). Thus, it is not a coincidence that the specific patterns of sulfation, the glycosidic linkage, the branching and the type of sugar have central roles in triggering the acrosome reaction during fertilization (Vilela-silva et al. 2008; Pomin 2015). Similarly, when tested for pharmacological activities, they usually have effects that differ according to their structures.

The analysis of the biological activity of the three polysaccharides studied here has raised many interesting questions. For instance, the sulfated fucan from *S. franciscanus* and the sulfated galactan from *E. lucunter* have the same sulfation pattern (2-O-sulfated), glycosidic linkage (1→3), and α anomeric configuration. However, the single difference in the sugar type results in a significant difference in their anticoagulant properties. The fucan from *S. franciscanus* is approximately 10 times less effective as an anticoagulant than the *E. lucunter* galactan, as indicated by aPTT values. Moreover, the effect of the fucan is exclusively based on its potentiation of the antithrombin-mediated

factor Xa inhibition. This shows that this single difference is sufficient to dictate how the polysaccharide interacts with many different proteins (Pereira et al. 2002). In further work using molecular dynamics and docking protocols, these two molecules seemed to have similar conformations in solution but different binding orientations in the anticoagulant ternary complexes composed by the oligosaccharide, thrombin and antithrombin (Becker et al. 2007). Another example is the acrosome reaction. Although the sulfated fucans from *S. franciscanus* and *S. droebachiensis* are structurally almost identical, differing only in the glycosidic linkage (1→3 and 1→4, respectively), they are not able to induce interspecies acrosome reactions (Hirohashi et al. 2002). This suggests a requirement for a specific glycosidic linkage on the 2-O-sulfated fucan. On the other hand, cross-acrosome reaction between two other fucans from the sea urchins *Strongylocentrotus purpuratus* and *Strongylocentrotus pallidus* has been observed. In this case, the two species possess α(1→3) linked sulfated fucans with different sulfation patterns, but this feature alone could not impair the induction of acrosome reaction between species (Vilela-silva et al. 2008). This cross-acrosome reaction is also observed between the fucan from *S. franciscanus* and the galactan from *E. lucunter*, which shows that the single difference in the sixth carbon between the two molecules is not important for proper recognition by the sperm receptors (Hirohashi et al. 2002).

Whereas the fucan from *S. droebachiensis* and the galactan from *E. lucunter* could prevent the formation of experimental metastasis and inflammation-mediated leukocyte recruitment, the fucan from *S. franciscanus* lacks any activity. In this case, a complex set of features was necessary to ensure anti-selectin activity. The only difference between active galactan from *E. lucunter* and the inactive fucan from *S. franciscanus* is the missing hydroxyl group on carbon 6 in the fucan. However, the different linkage of the sulfated fucan 1→4 found in *S. droebachiensis* restores the antimetastatic activity. One possible explanation for this difference is that the three-dimensional conformation alters the ligand presentation between these polysaccharides. The fucan from *S. franciscanus* and the galactan from *E. lucunter* are known to have similar 3D conformations (Becker et al. 2007), but a recent NMR study showed that they have slightly differential dynamics. Specifically, the α-L-fucan is a more flexible molecule in solution, and this can relate to their differential binding properties (Queiroz et al. 2016).

Regarding the antimetastatic activity, we can infer that the *S. droebachiensis* fucan prevented metastasis most efficiently in our model. The *E. lucunter* galactan, however, was a slightly better inhibitor because the *S. droebachiensis* fucan has a higher anticoagulant activity, which could be associated with undesirable side effects during treatment. Additionally, the galactan's higher efficacy in inflammation (Figure 5) could be explained by the interaction with other molecules such as L-selectin, integrins or chemokines (Marki et al. 2015).

Finally, in this work, we presented a new application for fucans and galactans on the basis of their demonstrated potential to inhibit P-selectin and prevent metastasis and cell recruitment during inflammation. We also showed that the structure-function relationship between them is complex, and future studies within this context might provide new insights for the glycobiology field, as specific features of these sulfated polysaccharides directly regulated several pharmacological effects.

Materials and methods

Cell lines and reagents

Human colon carcinoma cells (LS180; ATCC, Manassas, VA, USA) were grown in minimum essential medium-α (MEM-α) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Lewis lung adenocarcinoma cells (LLC; ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) (Vitrocell) supplemented with 10% FBS. All reagents were from Sigma (St Louis, MO, USA), unless otherwise stated. UFH (Liquemine) was obtained from Roche Pharma (Reinach, Switzerland).

Isolation of the sulfated fucans and galactan from sea urchins

Echinometra lucunter specimens were collected at Guanabara Bay, Rio de Janeiro, Brazil, and the egg jelly was obtained as described (Vacquier and Moy 1997). The egg jelly from *S. droebachiensis* and *S. franciscanus* were kindly provided by professor Christiane Biermann (Portland State University, USA) and professor Victor Vacquier (University of California San Diego, USA) respectively. Polysaccharide purification was performed by anion exchange chromatography as previously described (Vilela-Silva et al. 1999; Vilela-Silva et al. 2002).

P-selectin inhibition assay in vitro

Calcein-AM-labeled LS180 cells and increasing concentrations of the sulfated polysaccharides were added in triplicate to a 96-well plate with immobilized P-selectin chimeras, as described previously (Hostettler et al. 2007).

Animal models

An animal use protocol was obtained (05/2016), and procedures were followed in strict accordance with guidelines established by the Comissão de Ética no Uso de Animais / Instituto de Pesquisas Biomédicas / Hospital Naval Marcílio Dias (CEUA-IPB).

Platelet-tumor cell association in vivo

Lung sections were obtained from the mice 30 minutes after the intravenous injection of the tumor cells as described previously (Borsig et al. 2001), with slight modifications. Briefly, LLC cells were labeled with calcein-AM and intravenously injected into C57BL/6 mice with or without previous intravenous injection of 100 µg of the sulfated polysaccharides. After 30 minutes, the lungs were harvested for analysis. The lung sections were immunofluorescently stained with goat anti-integrin αIIb (CD41) (Santa Cruz Biotechnology) followed by anti-goat Cy3-conjugated antibody and analyzed by immunofluorescence microscopy. The platelet-tumor cell association was quantified by evaluating the calcein-AM labeled cells present in 10 fields of each lung section (10 sections per animal, 3 animals per experimental group).

Experimental metastasis model

C57BL/6 mice were intravenously injected with 10^6 LLC cells via the tail vein. Mice were injected either with PBS (vehicle) or 100 µg of the sulfated polysaccharides 15 minutes prior to tumor cell injection and killed after 21 days. PBS perfused lungs were macroscopically evaluated for the presence of metastatic foci.

Thioglycolate-induced acute peritonitis

C57BL/6 mice were intravenously injected with 100 µg of the sulfated polysaccharides. After 15 minutes, the animals were injected intraperitoneally with 1 mL of 4% thioglycolate. The mice were sacrificed after 3 hours, and peritoneal lavage was collected using 8 mL ice-cold PBS. This lavage was analyzed after cytospin centrifugation and staining with Wright's stain. Differential counting was performed to evaluate the percentage of polymorphonuclear cells present in the peritoneal cavity.

Funding

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Ary Frauzino para Pesquisa e Controle do Câncer.

Acknowledgements

The authors would like to thank Professor Eliene Kozlowski (*in memoriam*), who participated from the beginning of the project design to the data analysis of all experiments in this paper. The authors would also like to thank professor Mariana Stelling for substantial revisions to the manuscript and the students Mariana Soares and Tamires Gerhardt for technical support.

Abbreviations

Fucopyranose (Fuc*p*), galactopyranose (Gal*p*), sialyl-lewis^{x} (sLe^{x}), sialyl-lewis^{a} (sLe^{a}), glycosaminoglycan (GAG), phosphate-buffered saline (PBS), polymorphonuclear leukocytes (PMN), activated partial thromboplastin time assay (aPTT), unfractionated heparin (UFH), Lewis lung carcinoma (LLC), fetal bovine serum (FBS), extracellular matrix (ECM).

References

Becker CF, Guimarães JA, Mourão PAS, Verli H. 2007. Conformation of sulfated galactan and sulfated fucan in aqueous solutions: implications to their anticoagulant activities. *J. Mol. Graph. Model*. 26:391–399.

Borsig L. 2010. Antimetastatic activities of heparins and modified heparins. Experimental evidence. *Thromb. Res*. 125, Supplement 2:S66–S71.

Borsig L, Wong R, Feramisco J, Nadeau DR, Varki NM, Varki A. 2001. Heparin and cancer revisited: Mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. *Proc. Natl. Acad. Sci*. 98:3352–3357.

Borsig, L., Jennifer L. Stevenson, J.L., and Varki, A. (2007). Heparin in Cancer: Role of Selectin Interactions. In: A.A. Khorana, and C. Francis, editors. *Cancer-Associated Thrombosis*. (New York: Informa Healthcare), pp. 97–113.

Brown JR, Fuster MM, Whisenant T, Esko JD. 2003. Expression Patterns of α2,3-Sialyltransferases and α1,3-Fucosyltransferases Determine the Mode of Sialyl Lewis X Inhibition by Disaccharide Decoys. *J. Biol. Chem*. 278:23352–23359.

Fernando IPS, Nah J-W, Jeon Y-J. 2016. Potential anti-inflammatory natural products from marine algae. *Environ. Toxicol. Pharmacol*. 48:22–30.

Fitton JH, Stringer DN, Karpiniec SS. 2015. Therapies from Fucoidan: An Update. *Mar. Drugs* 13:5920–5946.

Gomes, A.M., Kozlowski, E.O., Borsig, L., Teixeira, F.C.O.B., Vlodavsky, I., and Pavão, M.S.G. 2015. Antitumor properties of a new non-anticoagulant heparin analog from the mollusk Nodipecten nodosus: Effect on P-selectin, heparanase, metastasis and cellular recruitment. *Glycobiology* 25:386–393.

Hirohashi N, Vilela-Silva A-CES, Mourão PAS, Vacquier VD. 2002. Structural requirements for species-specific induction of the sperm acrosome reaction by sea urchin egg sulfated fucan. *Biochem. Biophys. Res. Commun*. 298:403–407.

Hostettler N, Naggi A, Torri G, Ishai-Michaeli R, Casu B, Vlodavsky I, Borsig L. 2007. P-selectin- and heparanase-dependent antimetastatic activity of non-anticoagulant heparins. *FASEB J*. 21:3562–3572.

Kansas GS. 1996. Selectins and their ligands: current concepts and controversies. *Blood* 88:3259–3287.

Kim YJ, Borsig L, Varki NM, Varki A. 1998. P-selectin deficiency attenuates tumor growth and metastasis. *Proc. Natl. Acad. Sci*. U. S. A. 95:9325–9330.

Kim YJ, Varki A. 1997. Perspectives on the significance of altered glycosylation of glycoproteins in cancer. *Glycoconj. J*. 14:569–576.

Kozlowski, E.O., Pavao, M.S.G., and Borsig, L. 2011. Ascidian dermatan sulfates attenuate metastasis, inflammation and thrombosis by inhibition of P-selectin. *J. Thromb. Haemost*. 9:1807–1815.

Leblanc R, Peyruchaud O. 2016. Metastasis: new functional implications of platelets and megakaryocytes. *Blood* 128:24–31.

Ludwig RJ, Schön MP, Boehncke W-H. 2007. P-selectin: a common therapeutic target for cardiovascular disorders, inflammation and tumour metastasis. *Expert Opin. Ther. Targets* 11:1103–1117.

Marki A, Esko JD, Pries AR, Ley K. 2015. Role of the endothelial surface layer in neutrophil recruitment. *J. Leukoc. Biol*. 98:503–515.

Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD. 1993. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell* 74:541–554.

Mourão PAS. 2015. Perspective on the use of sulfated polysaccharides from marine organisms as a source of new antithrombotic drugs. *Mar. Drugs* 13:2770–2784.

Mulloy B, Ribeiro AC, Alves AP, Vieira RP, Mourão PA. 1994. Sulfated fucans from echinoderms have a regular tetrasaccharide repeating unit defined by specific patterns of sulfation at the 0-2 and 0-4 positions. *J. Biol. Chem*. 269:22113–22123.

Pavão MS, Albano RM, Lawson AM, Mourão PA. 1989. Structural heterogeneity among unique sulfated L-galactans from different species of ascidians (tunicates). *J. Biol. Chem*. 264:9972–9979.

Pavão MSG. 2014. Glycosaminoglycans analogs from marine invertebrates: structure, biological effects, and potential as new therapeutics. *Front. Cell. Infect. Microbiol*. 4:123.

Pereira MS, Melo FR, Mourão PAS. 2002. Is there a correlation between structure and anticoagulant action of sulfated galactans and sulfated fucans? *Glycobiology* 12:573–580.

Pomin VH. 2012. Fucanomics and galactanomics: Current status in drug discovery, mechanisms of action and role of the well-defined structures. *Biochim. Biophys. Acta BBA - Gen. Subj*. 1820:1971–1979.

Pomin VH. 2015. Sulfated glycans in sea urchin fertilization. *Glycoconj. J*. 32:9–15.

Pomin VH, Mourão PAS. 2008. Structure, biology, evolution, and medical importance of sulfated fucans and galactans. *Glycobiology* 18:1016–1027.

Queiroz INL, Vilela-Silva A-CES, Pomin VH. 2016. Oligosaccharides from the 3-linked 2-sulfated alpha-L-fucan and alpha-L-galactan show similar conformations but different dynamics. *Glycobiology* 26:1257–1264.

Ruggeri ZM, Mendolicchio GL. 2007. Adhesion Mechanisms in Platelet Function. *Circ. Res*. 100:1673–1685.

Schumacher D, Strilic B, Sivaraj KK, Wettschureck N, Offermanns S. 2013. Platelet-Derived Nucleotides Promote Tumor-Cell Transendothelial Migration and Metastasis via P2Y2 Receptor. *Cancer Cell* 24:130–137.

Vacquier VD, Moy GW. 1997. The Fucose Sulfate Polymer of Egg Jelly Binds to Sperm REJ and Is the Inducer of the Sea Urchin Sperm Acrosome Reaction. *Dev. Biol*. 192:125–135.

Valastyan S, Weinberg RA. 2011. Tumor Metastasis: Molecular Insights and Evolving Paradigms. *Cell* 147:275–292.

Vilela-Silva AC, Alves AP, Valente AP, Vacquier VD, Mourão PA. 1999. Structure of the sulfated alpha-L-fucan from the egg jelly coat of the sea urchin Strongylocentrotus franciscanus: patterns of preferential 2-O- and 4-O-sulfation determine sperm cell recognition. *Glycobiology* 9:927–933.

Vilela-Silva A-CES, Castro MO, Valente A-P, Biermann CH, Mourão PAS. 2002. Sulfated Fucans from the Egg Jellies of the Closely Related Sea Urchins Strongylocentrotus droebachiensis and Strongylocentrotus pallidus Ensure Species-specific Fertilization. *J. Biol. Chem.* 277:379–387.

Vilela-silva A-CES, Hirohashi N, Mouro PAS. 2008. The structure of sulfated polysaccharides ensures a carbohydrate-based mechanism for species recognition during sea urchin fertilization. *Int. J. Dev. Biol*. 52:551–559.

Zeeshan R, Mutahir Z. 2017 Mar 9. Cancer metastasis - tricks of the trade. Bosn. J. Basic Med. Sci.

Figure legends

Figure 1. **Structures of the 2-O-sulfated α-L-fucans and α-L-galactan from different species of sea urchins.** The three fully characterized structures of the sulfated polysaccharides isolated from the egg jelly coats of sea urchins. They show the same sulfation pattern but differ with respect to the glycosidic linkages and the constituent monosaccharides.

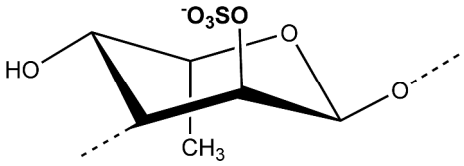
Figure 2. ***In vitro*** **tumor cell binding to immobilized P-selectin is inhibited by sea urchin sulfated polysaccharides.** The LS180 cell adhesion to immobilized P-selectin chimeras was measured in the presence of increasing concentrations of unfractionated heparin; *E. lucunter* 1→3 linked galactan; *S. franciscanus* 1→3 linked fucan; and *S. droebachiensis* 1→4 linked fucan. Each curve represents 3 independent experiments. The IC_{50} values, expressed in μg/mL, were 24,5 for UFH; 9,6 for the *E. lucunter* galactan; 170 for the *S. franciscanus* fucan and 11 for the *S. droebachiensis* fucan.

Figure 3. **Inhibition of LLC cells binding to platelets *in vivo* by the sulfated polysaccharides** (A) Representative images of platelet (red)-tumor cell (green) association in lung sections (DNA in blue) 30 minutes after injection of the tumor cells into mice that had been pre-treated with the *S. franciscanus* fucan, the *S. droebachiensis* fucan, the *E. lucunter* galactan or PBS; (B) $3x10^5$ LLC cells were injected into C57BL/6 mice after previous injection of 100 μg per animal of the polysaccharides or injection of PBS as a control group. *** $p<0.001$ ** $p<0.01$.

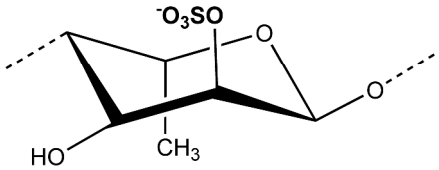
Figure 4. **Sea urchin sulfated polysaccharides attenuate experimental metastasis of LLC adenocarcinoma cells.** A total of 10^6 LLC cells were injected into C57BL/6 mice that had been pre-treated with 100 μg per mouse of the following compounds: PBS (Control); the *S.*

franciscanus fucan; the *S. droebachiensis* fucan; or the *E. lucunter* galactan. After 21 days, the mice were euthanized and perfused with PBS, and the macrometastatic foci were quantified. *** $p<0.001$.

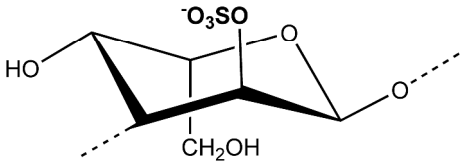
Figure 5. **Polymorphonuclear leukocyte recruitment in a thioglycolate-induced peritonitis model is inhibited by sea urchin polysaccharides.** C57BL/6 mice were intraperitoneally injected with 1 mL of thioglycolate (4%) 15 minutes after intravenous injection of the *S. franciscanus* fucan, the *S. droebachiensis* fucan, the *E. lucunter* galactan or PBS. The negative control group was injected with PBS only. After 3 hours, we performed a peritoneal wash and the percentages of polymorphonuclear leukocytes (PMN) were obtained based on cell morphology. (A) The cells in the peritoneal wash were stained with Wright's Stain and differentially counted for mononuclear and polymorphonuclear cells. (B) Percentage of PMN cells per group. Statistical significance was determined using ANOVA. *** $p<0.001$.



Fucan 1→3 linked
Strongylocentrotus franciscanus



Fucan 1→4 linked
Strongylocentrotus droebachiensis



Galactan 1→3 linked
Echinometra lucunter

Figure 1. Structures of the 2-O-sulfated α-L-fucans and α-L-galactan from different species of sea urchins. The three fully characterized structures of the sulfated polysaccharides isolated from the egg jelly coats of sea urchins. They show the same sulfation pattern but differ with respect to the glycosidic linkages and the constituent monosaccharides.

180x368mm (600 x 600 DPI)

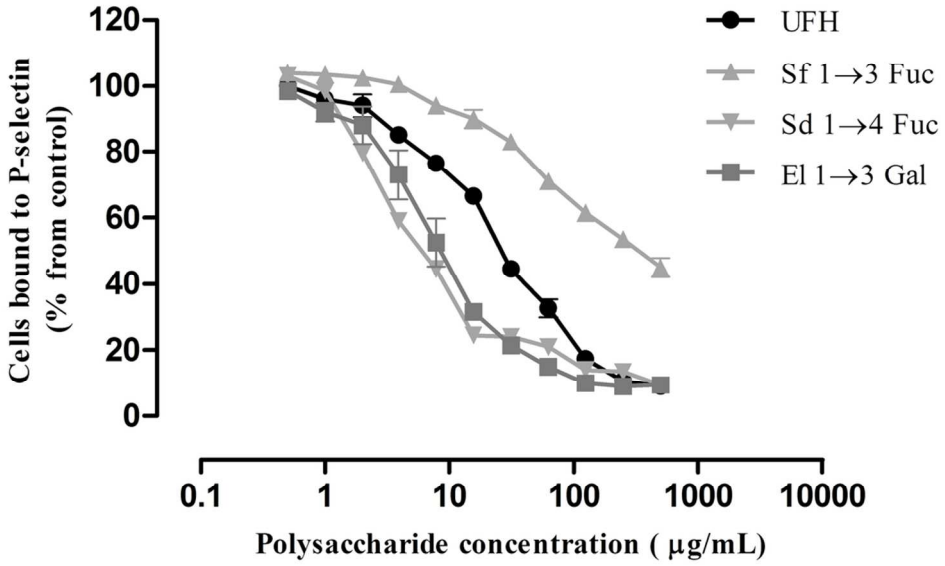


Figure 2. In vitro tumor cell binding to immobilized P-selectin is inhibited by sea urchin sulfated polysaccharides. The LS180 cell adhesion to immobilized P-selectin chimeras was measured in the presence of increasing concentrations of unfractionated heparin; E. lucunter 1→3 linked galactan; S. franciscanus 1→3 linked fucan; and S. droebachiensis 1→4 linked fucan. Each curve represents 3 independent experiments. The IC50 values, expressed in µg/mL, were 24,5 for UFH; 9,6 for the E. lucunter galactan; 170 for the S. franciscanus fucan and 11 for the S. droebachiensis fucan.

50x32mm (600 x 600 DPI)

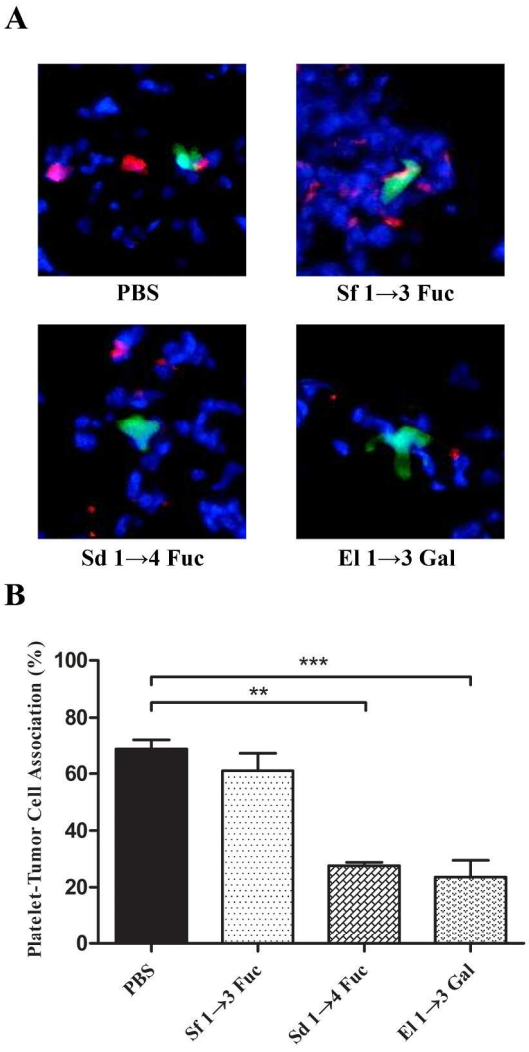


Figure 3. Inhibition of LLC cells binding to platelets in vivo by the sulfated polysaccharides (A) Representative images of platelet (red)-tumor cell (green) association in lung sections (DNA in blue) 30 minutes after injection of the tumor cells into mice that had been pre-treated with the S. franciscanus fucan, the S. droebachiensis fucan, the E. lucunter galactan or PBS; (B) 3x105 LLC cells were injected into C57BL/6 mice after previous injection of 100 µg per animal of the polysaccharides or injection of PBS as a control group. *** $p<0.001$ ** $p<0.01$.

180x368mm (300 x 300 DPI)

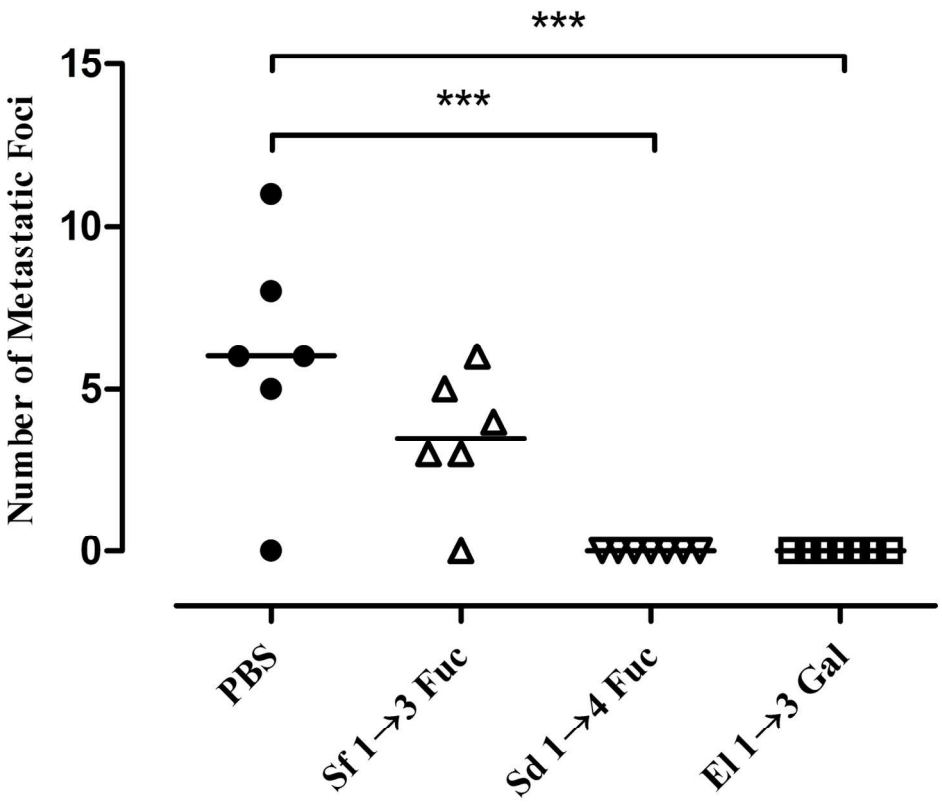


Figure 4. Sea urchin sulfated polysaccharides attenuate experimental metastasis of LLC adenocarcinoma cells. A total of 106 LLC cells were injected into C57BL/6 mice that had been pre-treated with 100 µg per mouse of the following compounds: PBS (Control); the S. franciscanus fucan; the S. droebachiensis fucan; or the E. lucunter galactan. After 21 days, the mice were euthanized and perfused with PBS, and the macrometastatic foci were quantified. *** $p<0.001$.

68x59mm (600 x 600 DPI)

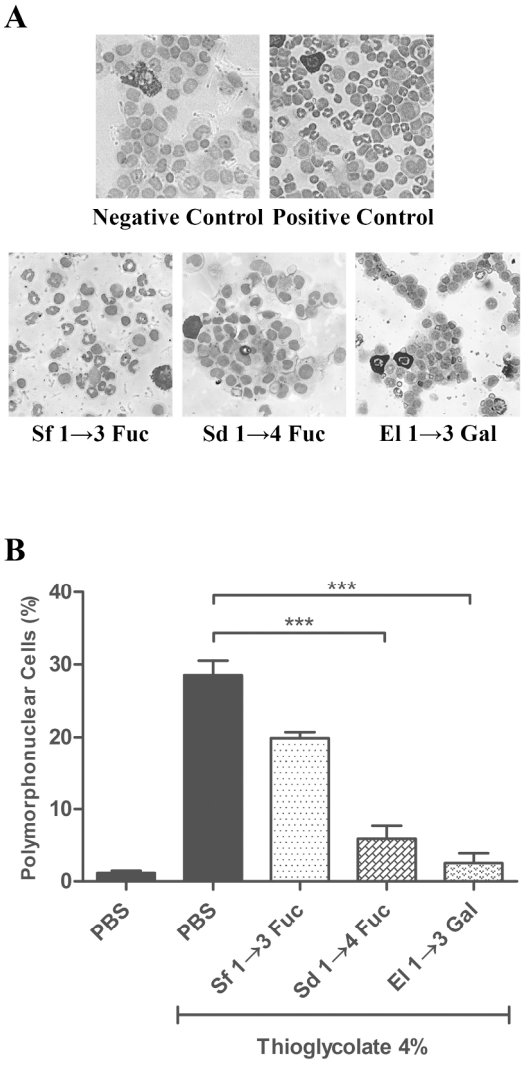


Figure 5. Polymorphonuclear leukocyte recruitment in a thioglycolate-induced peritonitis model is inhibited by sea urchin polysaccharides. C57BL/6 mice were intraperitoneally injected with 1 mL of thioglycolate (4%) 15 minutes after intravenous injection of the S. franciscanus fucan, the S. droebachiensis fucan, the E. lucunter galactan or PBS. The negative control group was injected with PBS only. After 3 hours, we performed a peritoneal wash and the percentages of polymorphonuclear leukocytes (PMN) were obtained based on cell morphology. (A) The cells in the peritoneal wash were stained with Wright's Stain and differentially counted for mononuclear and polymorphonuclear cells. (B) Percentage of PMN cells per group. Statistical significance was determined using ANOVA. *** $p<0.001$.

180x368mm (300 x 300 DPI)